Use of Soluble Protein Molecules Expressed by the Pancreas and Kidney Glomerulus

The Technical Field of the Invention

The present invention is related to soluble nephrin-like protein molecules as well as nucleic acid sequences having a substantial similarity with SEQ ID NO:1: and which encode nephrin-like protein molecules which are substantially homologous with human nephrin (SEQ ID NO:2:) but lacking the transmembraneous domain (SEQ ID NO: 3:) thereof. Said nephrin-like molecules and the nucleic acid sequences encoding them are useful for diagnostic determination, prophylactic and therapeutic treatment of diabetic and other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The Background of the Invention

The NPHS1-gene and its non-soluble gene-product, named nephrin, found abnormal in patients suffering from congenital nephrotic syndrome of the Finnish type (CNF), an autosomal-recessive disorder characterized by massive proteinuria in utero and nephrosis at birth, has been described by Kestilä, M., et al. (Molecular Cell 1: 575-582, 1998). The NPHS1 gene product is a non-secretable 1241-residue putative transmembrane protein with resemblance to immunoglobulin family of cell adhesion molecules, and exclusively expressed in kidney as reported by Kestilä, M., et al, 1998. The nucleotide derived amino-acid sequence of NPHS1 and its predicted domain structure has also been described in said publication.

Patent publication WO 99/47562 discloses the use of nucleic acids and proteins of said non-secretable, non-soluble form of nephrin for diagnoses and small molecule therapy of basement membrane disease. The present inventor has identified a new splicing form of the said nephrin encoding mRNA, which is translated into a secretable, soluble nephrin-like molecule. The nephrin-like molecule is expressed and secreted by both kidney and pancreas, indicating that it is not exclusively expressed by kidney. Based on the discovery of this new tissue expression pattern, the present inventor has been able to develop new methods and means for studying, e.g. diabetic and other nephropathies (glomerulopathy), which is one of the most common complications of prolonged diabetes. The treatment of the disorder is also complicated with still undetermined treatment modalities. The ultimate treatment of this and other serious nephropathies with

kidney transplantation, is complex, expensive, risky and binds the patients to continuous use of immunomodulatory medication.

Even if it is known that diabetes mellitus is a major cause of end-stage kidney disease, the pathophysiology of especially the glomerular permeability changes in diabetes mellitus remain poorly understood, and little progress has been achieved, in addition to the strict glycemic control, in the targeted treatment of this symptom. Simultaneously with the changing demographics and continuous increase in the incidence of diabetes mellitus, an ever increasing economic burden to health care organizations in all western countries will be evident. Thus, there is a great need of methods and test kits for accurate diagnosis of said disorder as well as methods and means for prophylactic, preventive and therapeutic treatment thereof. In addition, this same applies for other either primary or secondary diseases of the kidney presenting with proteinuria. Moreover, inflammatory, neoplastic, developmental and other acquired diseases of the exocrine and endocrine pancreas have remained poorly characterized often with no known etiopathogenesis.

Thus, the objectives of the present invention is to provide new methods and means for diagnostic determination, for prophylactic and therapeutic treatment, for evaluating the efficacy of treatment and for screening large populations of the susceptibility of diabetic and other nephropathies and of diabetes mellitus and other inflammatory endocrine and neoplastic pancreatic diseases based on the use of the soluble nephrin-like molecules of the present invention, including the splice variant and derivatives of said molecules as well as nucleic acid sequences encoding them.

The present invention provides a solution to the above defined problems by offering new methods and means for diagnosing as well as for prophylactic and therapeutic treatment based on the use of soluble nephrin-like molecules and nucleic acid sequences encoding the same.

The Summary of the Invention

The characteristic features of the methods and means are defined in the claims of the present invention.

A Short Description of the Drawings

Figure 1 depicts alignment of nucleotide-derived amino acid sequences of rat and human nephrin. The putative transmembrane domain appears in the box, Ig-like modules are

marked by shadowing and fibronectin type III-like module is underlined. Cysteine residues identical in human and rat are shown by closed dots and the two additional cysteines of rat are indicated by arrows (an additional cysteine is found in human locus 974). The cleavage site for predicted N-terminal signal sequence is identical for human and rat as are the putative N-glycosylation sites (not marked).

Figure 2 depicts amino acid sequence of nephrin and the respective NPHS1 nucleotides 3121 to 3300 including the transmembrane area (boxed) together with exon boundaries. The missing sequence of the novel nephrin-α splicing variant includes exon 24.

Figure 3 depicts immunoblotting of glomerular lysates with antinephrin antibodies shows distinct reactivity with a 200 kD protein band.

Figure 4 depicts consequtive patient serum samples taken after renal transplantation before renephrosis.

Figure 5 depicts kidney biopsies from normal kidneys (Figure 5A, Figure 5B) and diseased kidneys (Figure 5C and Figure 5D).

Figure 6 depicts expression of nephrin mRNA in different human tissues. Hybridization signals are seen only in adult (EI) and fetal (G3) kidney as well as in pancreas (D3).

Figure 7 depicts RT-PCR of nephrins found in islets of Langerhans.

Figure 8 depicts Western blots of nephrins from kidney cortex and pancreas.

Figure 9 depicts paraffin sections of mouse pancreas.

The Detailed Description of the Invention

Definitions

In the present invention the terms used have the meaning they generally have in the fields of medicine and diagnostics, especially in bed-side diagnostics as well as immunochemistry. Some terms, however, are used with a somewhat deviating or broader meaning in this context. Accordingly, in order to avoid uncertainty caused by terms with unclear meaning some of the terms used in this specification and in the claims are defined in more detail below.

In the present invention the term "**nucleic acid sequence**" means an isolated nucleic acid sequence encoding nephrin or soluble nephrin-like molecules shared by pancreas and kidney glomerulus and having the "**nucleic acid sequences**" comprise SEQ ID NO:1: or nucleic acid sequences with substantial similarity encoding nephrin-like molecules having an amino acid sequence substantially homologous with SEQ ID NO:2: but lacking the transmembraneous domain GPSGLPLLPVLFALGGLLLLSNASCVGGVL-WQRRLRRL (SEQ ID NO:3:) of nephrin or substantial parts therof. Preferably the nucleic acid should encode a polypeptide having the characteristics described above and at least one contagious amino acid sequence LPTEPPSGAEGISE (SEQ ID NO:4:) surrounding the transmembraneous domain. The overlapping sequence can be longer or shorter than said SEQ ID NO:4: and/or it can in addition to the transmembraneous domain lack one or more, preferably three or more amino acid sequences from the left or right side of the transmembraneous domain. The most important characteristic feature of the nucleotide and/or amino acid sequence of the present invention being the solubility, which provides possibilities to detect the protein from blood, serum and/or urine samples as well as from tissue fluids. In other words the protein is not basement membrane bound. Because the transmembraneous domain is missing and domains from both sides or either side of the transmembraneous domain, i.e. intra- and extracellular domains, are present, the probability of finding such very specific regions are highest in the vicinity of the missing domain or area.

The "**nucleic acid sequences**" of the present invention are not in their natural state but are isolated from their natural environment as expressed mRNAs, which are purified and multiplied *in vitro* in order to provide by technical means new copies, which are capable of encoding said human nephrin or substantially homologous "**soluble nephrin-like molecules**" of the present invention.

The isolated nucleic acid sequences of the present invention also include the human nephrin encoding nucleic acid sequence SEQ ID NO:1:, which is obtainable as a cDNA of mRNA expressed by human pancreas and kidney glomerulus. Said sequence differs from the mutation form of NPHS1 gene described by Kestilä et al. (1998) and in WO 99/47562 by having at least one amino acid substituted with another amino acid, e. g. a "Leu" in the human locus or position 97 instead of "His" (corresponding to the human locus, i.e. position 75 in the sequence disclosed in the International Patent Application WO 99/47562), an "Ile" in position 273 instead of a "Leu" corresponding to human locus or position 251 in the International Patent Application WO 99/47562).

The term "**nucleic acid sequence encoding nephrin-like molecules**" means nucleic acid sequences as well as substantially homologous nucleic acid sequences, including genomic DNA, RNA and/or cDNA which comprise at least one contagious nucleic acid sequence, encoding the amino acid sequence, LPTEPPSAEGISE (SEQ ID NO:4:) overlapping the extra- and intracellular domains of the nephrin or nephrin-like molecules, but lacking the nucleic acid sequence encoding the transmembraneous domain GPSGLPLLPVL-FALGGLLLLSNASCVGGVLWQRRLRRL (SEQ ID NO:3:). As an example of such nucleic acid sequences the contagious sequence CTG CCC ACA GAG CCA CCT TCA GGC ATC TCA GAG (SEQ ID NO:5:) deduced from human cDNA can be mentioned. This sequence or its complementary sequence or nucleic acid sequences containing said sequence or parts thereof, e.g. fragments truncated at the 3'-terminal or 5'-terminal end as well as such sequences containing point mutations are especially useful as probes for detecting nucleic acid sequences of the present invention. Specific nucleic acid sequences useful as primers are the sequences for exon 2 comprise 5'-GAC AAA GCC AGA CAG ACG CAG-3' (SEQ ID NO:6:) and 5'-AGC TTC CGC (SEQ ID NO:7:) as well as other nucleotide sequences constructed from the known amino acid sequence. It is however clear for those skilled in the art that other nucleic acid sequence capable of encoding nephrin-like molecules and useful for their production can be prepared especially when taking in account the codon degeneracy and varying the amount of triplets taken in consideration on either side of the nucleic acid sequence encoding the transmembrane domain. The nucleic acid sequences encoding nephrin-like molecules should not be capable of hybridizing under stringent condition ((Sambrook, J., et al., Molecular Cloning: A Laboratory Manual., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989) with sequence encoding the transmembrane domain of the nephrin or parts thereof.

The nucleic acid sequences of the present invention should have a substantial similarity with the SEQ ID NO:1:. "**Substantial similarity**" means that the nucleotide sequences fulfill the prerequisites defined above and have a significant similarity, i.e. a sequence identity of at least 60 %, preferably 70 %, most preferably more than 80 % with the regions of SEQ ID NO:1:, encoding the intra- and extracellular domains, but lacking the region encoding the transmembraneous domain of human nephrin.

The term "**nucleic acid sequences encoding human nephrin or soluble nephrin-like molecules**" include their truncated or complexed forms as well as point mutations of said nucleic acid sequences as long as they are capable of encoding amino acid sequences

having the essential structural features as well as the properties and/or functions of nephrin-like molecules.

The term "**nephrin-like molecules**" means protein molecules or polypeptides, i.e. soluble receptor analogues expressed by pancreas and kidney glomerulus and being substantially homologous to nephrin at amino acid level, but lacking the transmembrane domain of nephrin. Said "**nephrin-like molecules**" are obtainable by isolation from natural sources as transmembrane splicing forms of the nephrin. The splicing forms lack the transmembrane anchoring domain of nephrin and the corresponding mRNA and cDNA lacks said transmembraneous domain encoding nucleic acid sequence or is a nucleic acid sequence from which said region is easily split off. The nephrin-like molecules are also producible by synthetic, semisynthetic, enzymatic and other biochemical or chemical methods including recombinant DNA techniques.

The "**nephrin-like molecules**" are substantially homologous with the amino acid sequence SEQ ID NO:2: but lacking the transmembraneous domain, i.e. at least the amino acid sequence between the amino acid Glu position 1051 and amino acid Lys position 1100 or preferably the amino acid sequences between positions 1056 and 1093 GPSGLPLLPVLFALGGLLLLSNASCVGGVLWQRRLRRL (SEQ ID NO:3:).

The term "**substantially homologous**" at amino acid level means that the nephrin-like protein molecules have a significant similarity or identity of at least 80%, preferably 85 %, most preferably more than 90 % with human nephrin (SEQ ID NO:2:) but lacks the transmembraneous domain (SEQ ID NO:3:).

The term "**nephrin-like molecules and derivatives thereof**" comprise polypeptides having the structure, properties and functions characteristic of nephrin-like molecules. Thus, the term "**nephrin-like molecules and derivatives thereof**" includes nephrin-like molecules, wherein one or more amino acid residues are substituted by another amino acid residue. Also truncated, complexed or chemically substituted, forms of said nephrin-like molecules are included in the term "**nephrin-like molecules and derivatives thereof**". Chemically substituted forms include for example, alkylated, esterified, etherified or amidized forms with a low substitution degree, especially using small molecules, such as methyl or ethyl, as substituents, as long as the substitution does not disturb the properties and functions of the nephrin-like molecules. The truncated, complexed and/or substituted variants of said polypeptides are producible by synthetic or semisynthetic, including enzymatic and/or recombinant DNA techniques. The only other

prerequisite being that the derivatives still are substantially homologous with and have the properties and/or express the functions of the intra- and extracellular domains of nephrin, i.e. the soluble nephrin-like molecules of the present invention.

The "**properties**" and "**functions**" of the soluble nephrin and nephrin-like compounds are related to the regulation of proteinuria and is characterized by increased permeability in the kidney glomeruli. In contrast to the transmembraneous non-soluble nephrin, said soluble nephrin and nephrin-like compounds as well as their binding substances, including antibodies, especially autoantibodies which have been elicited by said soluble nephrins in the subject suffering from the diseases relevant for the present invention can be determined with *per se* known methods from blood, serum and/or urine samples. The fact that the soluble products of the present invention can be determined from blood, serum and/or urine is a specific characterizing feature of the present invention.

Preferably, all "**nephrin-like molecules and their derivatives**" should be recognizable using binding substances capable of recognizing the natural human nephrin or nephrin-like molecules. The term "**nephrin-like molecules**" otherwise covers all possible splice variants of nephrin expressed by pancreas and kidney. The term "molecules or proteins shared by pancreas and kidney" means that the protein is expressed by cells of both pancreatic and kidney tissue, it is they have the same tissue expression pattern.

As a conclusion "**nephrin-like molecules**" in its broadest aspect in the present invention, covers not only nephrin-like molecules derived from nature, including their isoforms of different origin, but also synthetically, semisynthetically, enzymatically produced nephrin-like molecules including molecules produced by recombinant DNA techniques. Said nephrin-like molecules can be used either as separate entities or in any combinations thereof.

The term "**isoform**" refers to the different forms of the same protein, which originate from different sources, e.g. different species. In the present invention the term, thus, includes fragments, complexes and their derivatives. For example, nephrin-like molecules can be generated by the cleavage of the proprotein. Different reactions, including different enzymatic and non-enzymatic reactions, proteolytic and non-proteolytic, are capable of creating a truncated, derivatized, complexed forms of the molecules.

The amount of nephrin-like molecules are preferably determined with "**binding substances**". The term "binding substance" means any substances capable of specifically

recognizing and binding the soluble nephrin-like molecule or derivatives therof or at least one portion in the intra- and/or extracellular domain of nephrin or both. Preferably, the binding substance should recognize simultaneously both domains of nephrin, but not the transmembrane anchoring domain. Such substances are, for example, receptors or binding proteins or peptides, capable of specifically binding said nephrin-like molecules, but above all they mean antibodies capable of specifically recognizing one or more nephrin-like molecules alone or in any combination. The antibodies include both polyclonal and/or monoclonal antibodies as well as fragments or derivatives thereof. Preferably, binding substances recognizing and binding sequence specific epitopes or active sites of the nephrin-like molecules should be chosen.

Said binding substances can be produced using the intra- and extracellular domains of nephrin or any nephrin-like molecules, their isomers as well as their fragments, derivatives and complexes with the prerequisite that they are lack the transmembraneous domain and are capable of acting as "**antigens**", in other words, antigens include any compositions or materials capable of eliciting an antibody response specific to said nephrin-like molecules. Said binding substances, preferably antibodies are producible by conventional techniques for producing polyclonal antibodies as well as monoclonal antibodies. The methods for preparing monoclonal antibodies include hybridoma techniques. Fragments of antibodies or other binding proteins like specific binding peptides can be developed by phage display techniques and produced by recombinant DNA techniques. All methods are well known by those skilled in the art and described in laboratory handbooks.

The term "**diagnosing**" means judging, predicting, assessing or evaluating from the recorded results if a person is susceptible of or suffers from diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases. The diagnoses also enable evaluation of the severity of the condition, therapy required as well as the efficacy of treatment modalities or medical treatment. Especially, early identification of diabetes in order to start prophylactic dietary or other treatments before the onset of the actual disease is a desirable feature, enabled by the present invention.

The results are recordable with means for performing immunoassays using nephrin-like molecules and/or their binding substances as well as parts thereof or means for performing amplification and hybridization methods using sequence specific probes or primers, which can be selected from the parts of SEQ ID NO:1: encoding the intra- and/or extracellular domains of human nephrin, preferably the one or both of the

extracellular.

The term "screening a population for the presence or absence of autoantibodies" means that based on the fact that subjects susceptible of diabetes mellitus and complications related to said disease produce antibodies against nephrin-like molecules, which can be determined using nephrin-like molecules, it is easy to screen a large population for the susceptibility of the diseases mentioned above from blood, serum and/or urine samples obtained from inflowing serum samples for routine diabetes diagnosis. If such autoantibodies can be detected in the serum of a person, it is a clear indication that the person in question is a potential diabetes patient and might be susceptible also to diabetic kidney diseases and should be treated appropriately.

The term "**immunoassay**" refers to a method or procedure capable of detecting and/or measuring at least one substance, either nephrin-like molecules or autoantibodies against said molecules using per se known means for performing an immunoassay, which means including a substance capable of specifically recognizing the substance to be determined, i.e. either at least one binding substance or a nephrin-like molecule or fragments thereof, for the desired application, respectively.

Well known examples of immunoassays are radioimmunoassays (RIA), radioimmunometric assays (IRMA), fluoroimmunometric assays (IFMA) enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), fluoroimmmunoassays (FIA), luminescence immunoassays, immunoagglutination assays, turbidimetric immunoassays, nephelometric immunoassays, etc. All methods are well known by those skilled in the art and described in laboratory handbooks.

The preparation and development of methods and means for measuring different antigens and antibodies, which can be applied also to the determination of nephrin-like molecules and their autoantibodies have been described for example in the following patent publications applicable as convenient bed-side kits US 5,591,645, US 5,712,170, US 5,602,040, US 5,622,871, US 5,656,503, EP 149 168, US 4,552,839, US 4,361,537, US 4,373,932, WO 86/04683, EP 154 749, EP 7654, WO 86/03839, EP 191 640, EP 212 599, US 4,552,839, EP 158 746, EP 225 054 and which are herewith incorporated by reference. Even if said patents are restricted to the development of test kits for diagnosing other diseases by aid of binding substance recognizing the respective active molecule, the man skilled in the art can use the information for developing corresponding test kits for measuring nephrin-like molecules of the present invention.

Any known immunochemical test methods and principles can be applied for diagnosing diabetic and other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases as well as for longitudinal or latitudinal screening of the progress of disease and effect of medical treatment. However, visual agglutination, flow-through and immunochromatographic methods are best suited for rapid bed-side assays or tests.

"**Bed-side assays**" refer to tests or procedures which can be carried out without any laboratory facilities and without the need of qualified laboratory personnel. Bed-side tests can be made by the physician, while the patients are visiting the doctors or when the doctors make their daily bed-side visits to the patients. "**Bed-side assays**" are preferably performed on "**solid carriers**" like test strips. Such bed-side assays are based on immunochemical and hybridization techniques and several applications have been developed and described in the literature. The only prerequisite for the skilled person to develop new methods and tools (test kits) is to select and thereafter provide suitable antigens, antibodies as well as parts therof for the immunoassays and suitable probes and primers for the hybridization and PCR-techniques. In the present invention such antigens, antibodies, probes and primers are disclosed.

The term "**prophylactic treatment**" includes specific dietary measure and/or precautions, e.g. glycemic control including possible medication, before the onset of diabetes. After the on-set of diabetes, "**prophylactic treatment**" requires therapeutic treatment of diabetes as a precaution in order to avoid kidney complications.

The term "**therapeutic treatment**" includes methods for treating persons with administration of the soluble protein product, gene therapy or preventing the genes causing the disease from expressing the gene products causing the diseases, including diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The General Description of the Invention

Renal involvement of poor glycemic control is a major complication of diabetes mellitus (Kanwar, Y.S., et al., Semin Nephrol 11:390-413,1991; Rennke, H.G.: Kidney Int 45:S58-63, 1994; Fioretto, P., et al., N Engl J Med 339:69-75, 1998). The diabetic nephropathy is characterized by a gradual and worsening loss of the glomerular filtration

barrier function, increase of glomerular basement membrane thickness and accumulation of amorphous material within the glomerular mesangium, composing the typical Kimmelstiel-Wilson pathology of glomeruli (Kanwar, Y.S., et al., Semin Nephrol 11:390-413,1991; Rennke, H.G., Kidney Int 45:S58-63, 1994.). Simultaneously with the loss of glomerular functions, deterioration of renal tubular functions has been found.

In spite of this, little is presently known of common epitopes shared with the kidney glomerulus and the islets of Langerhans of pancreas. Thus, the solid demonstration of success in the combined pancreas-renal transplantation comprises new evidence for the fact that shared functional epitopes are involved.

Results of the present inventor indicate that nephrin is distinctly down-regulated in the human proteinuric disease of congenital nephrotic syndrome and is dramatically down-regulated in the experimental puromycin model mimicking the human "minimal change" pure proteinuria (Luimula, P., et al., Kidney International, In Press). At the same time antipeptide antibodies to the respective protein product indicate that the protein is expressed in glomerular podocytes, the cells crucial for glomerular permeability (Ahola, H., et al., submitted). Surprisingly, the only other tissue site expressing both the gene and its protein product in a survey of 60 human cells and tissues was the islets of Langerhans. The initial results of the inventor indicated that both the gene and the respective protein is found and regulated also in insulin-producing cell lines (Palmen, T., et al., submitted) including the rat insulinoma cell RIN, AR42J and INS-cells. Furthermore, it was shown that antibodies to this gene product are characteristically found in both human diseases with proteinuria (Wang, et al., in preparation) and distinctly in prediabetics with high circulating antibody titres to glutamic acid decarboxylase (GAD65) and pancreatic Langerhans islet cell (ICA) antibodies (Palmen, T., et al., submitted).

Accordingly, the recently identified new tissue expression pattern and posttranscriptional splicing of the gene, which previously was believed to be uniquely expressed by kidney indicates that the gene product is expressed in a unique way only in the endocrine and exocrine cells of pancreas and kidney glomerulus. Antibodies raised against the protein product encoded by the gene undisputably shows that it exists primarily in pancreas and kidney. Approximately 60 % of the patients suffering from prediabetes (so called islet cell -and glutamic acid dehydrogenase-(GAD)-antibody positiveness) as well as patients suffering from certain kidney diseases produce antibodies against this molecule.

Mutations in the *NPSH1* gene are known to cause congenital nephrotic syndrome of the

Finnish type, a recessive autosomal disease characterized by massive proteinuria present already in utero. The NPHS1 gene product, nephrin, is a transmembrane protein and a putative member of the immunoglobulin superfamily. Recently nephrin was found to be critically involved in the regulation of glomerular permeability and shown to be located at the interpodocyte slit membranes. Here we report of an unexpected expression of nephrin mRNA and protein in the pancreas. The expression was verified with the human RNA Masterblot^{TM} and reverse transcriptase polymerase chain reaction. In Western blot, antibodies against intracellular and extracellular domains of nephrin detected a 165 kDa protein suggesting a distinct glycosylation pattern in the pancreas. Within the pancreas, nephrin was immunohistochemically localized specifically to the ß-cells of the islets of Langerhans, with similar results obtained using various nephrin antibodies. In conclusion, nephrin is a molecule shared apparently only with the insulin-producing pancreatic ß-cells and kidney glomerular podocytes. Preliminary results show that in islet cell antibody (ICA) positive prediabetic patients present with increased levels of antinephrin antibodies suggesting that pancreatic nephrin is an important autoantigen in the pathogenesis of insulin dependent diabetes mellitus.

In addition, the present inventor has identified a form of splicing of the nephrin coding mRNA which yields a secreted soluble receptor analogue of this protein in kidney and pancreas. Because the splicing variant is among the first proteins shared by the kidney and pancreas, it is especially promising for producing future diagnostic methods as well as for drug development and therapeutic treatments, such as the use of the soluble receptor analog to modify disease and for gene therapy. Based on said findings it is also possible to develop similar nephrin-like molecules and derivatives thereof which have the same properties and functions.

The characterization of the structure of the transmembraneous domain-less splice form of the gene and the clarification of its molecular function in kidney and pancreas has shown that the gene is critically involved in the regulation of kidney glomerular permeability barrier. NPHS1 and its respective gene product are present within the pancreatic acinar cells and in the islet cells of Langerhans. Extraordinarily, the kidney glomerulus and pancreas are the only cell types among the 60 different human tissue sites tested for positivity in a Northern blot for the specific mRNA expression. Furthermore, the present inventor has shown that this gene is distinctly expressed within various insulin producing cell lines (AR42J; RINm5F; INS-1) as well as in the isolated porcine and human pancreatic islands. The insulin secretagogue stimulation of the cell lines up-regulates nephrin expression. Furthermore, nephrin protein serves as an autoantigen

in a group of islet-cell antibody (ICA) and glutamic acid decarboxylase (GAD65) positive prediabetics and is similarly down-regulated in various proteinuric kidney diseases showing that a completely new functional molecule shared with the glomerular filtration barrier and functionally crucial of the pancreas is involved.

Based on the findings disclosed above it can be concluded that the nephrin-like molecules of the present invention, the nucleic acid sequences encoding them as well as the binding substances specifically recognizing them are highly promising molecules to understand the pathophysiology of e.g. diabetic nephropathy. The present invention provides isolated and essentially purified novel nucleic acid sequence defined above. The nucleic acid sequence covers at least part of the intracellular and the extracellular portion of the NPHS1-gene, but lacks the portion of the NPHS1-gene expressing the transmembrane anchoring portion of nephrin.

The nephrin-like molecules are soluble polypeptides or proteins and as such especially useful for detecting and/or determining the presence or absence of autoantibodies when screening people for susceptibility of diabetic and other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The binding substances, which are capable of specifically recognizing and binding the soluble nephrin-like molecules, are preferably such that are capable of specifically recognizing either at least one epitope in the intra- and/or the extracellular domain of nephrin or preferably an epitope covering both domains but not the transmembraneous domain.

The molecules described above, including nephrin-like molecules, nucleic acid sequences encoding them and binding substances specifically recognizing them are useful for diagnostic determination, prophylactic and therapeutic treatment of diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases as well as for screening a multitude of samples in order to evaluate if a person is susceptible of diabetic or other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The present invention is related to a diagnostic kit for determining whether a subject is a nephropathy disorder carrier or a patient having a nephropathy associated disorder, which kit includes at least one nephrin-like molecule capable of recognizing and specifically binding to autoantibodies from serum samples obtained from patients susceptible

of said diseases as well as packing means and instructions for use.

Alternatively, the invention is related to a diagnostic kit for determining whether a subject is a nephropathy disorder carrier or a patient having a nephropathy associated disorder, which kit includes at least one binding substance capable of recognizing and specifically binding to nephrin-like molecules from samples obtained from patients susceptible of said diseases as well as packing means and instructions for use.

The nephrin-like molecules and the corresponding autoantibodies can be assessed by various known methods. The levels of nephrin-like molecules in blood, serum and/or urine samples obtained from healthy and sick persons can be estimated using their specific antibodies. Autoantibodies and/or nephrin-like molecules can be identified by Western blotting. After SDS-PAGE of the preparations, the bands can be transferred onto nitrocellulose and characterized by staining with polyclonal antibodies specific to said nephrin-like molecules. With methods like this, specific results indicating correlation of severity of disease and medication needed can be obtained, but said methods are far too laborious and time consuming to be used routinely. Moreover, it is impossible to develop a rapid bed-side test based on any kind of electrophoresis. The diagnostic determination of the present invention should be aimed at evaluating the disease from a blood, serum and/or urine sample, in order to be able to give appropriate treatment regimen at the right time, as well as to monitor the effect of therapy and treatment modalities as well as the prognosis of the disease. A higher level of nephrin-like molecules means a more severe disease and the need of a more guarded prognosis and medical treatment as evidenced by the present invention. Specific therapy regimens are related to disease severity and actual disease activity.

The antibodies raised against nephrin-like molecules and being capable of specifically recognizing them, were used by the present inventor in immunoblot analysis of blood, serum and/or urine samples obtained from sick and healthy persons. Various representatives of poly- and monoclonal antibodies recognizing nephrin-like molecules listed below can be produced by *per se* known methods. Monoclonal antibodies of the present invention can be and have also been developed according to the original technique of Köhler and Milstein (Nature 256, 495, 1975).

Based on the results obtained and the antibodies available the present inventor developed new methods and test kits for an effective, rapid, and reliable assessment of the status and tissue destruction status as well as to identify the phases of disease activity in pancre-

as and kidneys of human beings. The methods and test kits of the present invention are based on the fact that there is a relation especially between the presence of nephrin-like molecules and autoantibodies against said nephrin-like molecules and susceptibility and the severity of disease activity. Also indicated is the fact that certain nephrin-like molecules alone or in any combination are more specific than others in assessing the disease and that there is some differences in specificity and selectivity, too. Hence, it is advantageous to develop test kits by which a multitude of nephrin-like molecules alone or in any combination could be determined simultaneously, either on the same test strip or on separate test strips. One or more of the binding substances or the fragments of soluble nephrin or nephrin-like molecules can be combined in a test strip or test kit in such a way that one or more of the soluble molecules having the intra- or extracellular domains are combined in such a way that the identification of the transmembrane-free nephrin or nephrin-like molecules is enabled. In preferred embodiments the nephrin-like molecules, shown to be most suitable or effective for a specific diagnostic purpose, were selected for the test kit, either alone or in any combination.

Different numeric results obtained in the experiments reflect the use of different sets of binding substances or antibodies as well as other variation in the test conditions. However, it is important to notice that even if the level of nephrin-like molecules differ in different persons, the ratio between diseased and healthy persons remains approximately the same and a qualitative, semiquantitative or even quantitative test can be developed for bed-side assessment of the severity of the diseases. The results can be recorded visually or by a recording instrument either directly or indirectly by adding a substrate capable of making the binding reaction recordable.

These findings indicate that an immunochromatographic test using monoclonal antibodies which capture and recognize nephrin-like molecules alone or in any combination, is fully sufficient for the diagnosing disease activity. The invention disclosed in the present patent specification provides a highly effective diagnostic tool for an accurate evaluation of the kind of treatment and regimen of therapy needed. The methods and test kits of the present invention also provide alternative bed-side diagnostic tools for evaluating the severity of the disease. At the same time the methods and test kits provide effective tools for follow up studies of the efficacy of the therapy or treatment as well as the dose-treatment response obtained.

Based on these founding methods and test kits were developed for diagnosing the level, and/or severity of the disease processes, evaluating the efficacy of drug treatments, other

treatment modalities, other medications and/or predicting the risk for progress of said diseases, wherein the detection is performed as a rapid and reliable immunological bed-side assay using blood, serum and/or urine samples from which one or more nephrin-like molecules alone and/or in combination can be measured.

The nucleic acid sequences of the present invention, which have been defined above, as including the intracellular and extracellular domains of SEQ ID NO:1: or alternatively defined as SEQ ID NO:1: lacking the transmembraneous domain (SEQ ID NO:3:) can be used to produce suitable primers and probes to be applied in *per se* known hybridization techniques and PCR-techniques. Many applicaple and feasible methods and techniques are described in literature, patent publications, laboratory handbooks and can be adapted to the purposes of the present invention. Some test kits for said methods are even commercially available and can be adapted for the purposes of the present invention. The PCR-techniques for amplifying, detecting and/or cloning nucleic acid sequences were first described and patented by Mullis, K.B. et al. for example in the European Patents EP 200 362 and EP 201 184.

Suitable primers and probes for amplification and/or nucleic hybridization techniques can be found among fragments and/or parts of the nucleic acid sequence SEQ ID NO:1:, excluding any parts hybridizing with the nucleic acid sequences encoding the trans-membraneous domain of nephrin. The nucleic acid sequence used as primers and probes should comprise at least 10 nucleotides complementary to 10, preferably 15, most preferably 20 consecutive nucleotides from the above-defined sequence.

The nucleic acid sequences defined above can be copied and inserted into suitable recombinant expression vector, capable of expressing the gene product when transferred into a suitable host which express and/or secrete, i.e. produce recombinant soluble nephrins and/or nephrin-like protein molecules useful in diagnostic kits. The nucleic acid sequences of the present invention are useful in methods for screening larger population groups in order to determine whether a person is a carrier of the gene causing the disorder, i.e. a nephropathy or associated disorder gene carrier. The method comprises detecting the presence or absence of the nucleic acid sequences of the present invention. Thus, the invention is related to a diagnostic test kit for amplifying a portion of a nucleic acid sequence, a pair of nucleic acid primers complementary to a portion of nucleic acid sequence defined above as well as packaging means and instructions for use.

Also targeting recombinant expression vector can be produced by inserting said nucleic

acid sequences and functionally inert modification thereof into the vectors. Such targeting vectors can be used in gene therapeutic applications. The targeting recombinant expression vectors are either modified so that they do not allow expression of nephrin-like molecules when integrated with the gene responsible for diabetic and other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

Accordingly, the present invention is related to a diagnostic kit for determining whether a subject is a nephropathy disorder gene carrier or a patient having a neuropathy associated disorder, which kit includes as probes nucleic acid sequences capable of hybridizing under stringent conditions to nucleic acid sequence describe above as well as packing means and instructions for use. The results are directly applicable to patient diagnostics, and the full diagnostic, prognostic and pharmacologic lead-compound identification dimensions can thus far only be speculated.

The invention enables development of a method for treating a patient afflicted with a nephropathy disorder which method comprises targeted administering a nucleic acid sequence and to a method for treating or preventing a diabetic and other nephropathies. The method comprises for example administering to a patient in need thereof a nucleic acid sequence capable of compensating or extinguishing the deficient action of the gene. The nucleotide sequence can be modified in many different known ways so as to act appropriately. Antisense-technology, knockout technology etc. can be used to provide the desired effect.

Despite the increased availability of genetically modified mouse strains, the experimental models in the rat have provided the most widely employed and versatile models to study diabetic and other nephropathies and diabetes mellitus and other inflammatory and neoplastic pancreatic diseases including their pathophysiology and functional genetics. The present inventor has cloned and characterized the rat nephrin cDNA. The rat cDNA has an open reading frame of 3705 bp, and shows 82 % sequence identity to the human nephrin cDNA and shows characteristic rat specific splicing variants. The translated nucleotide sequence has 89 % sequence identity at the amino acid level. The signal sequence, glycosylation and cystein localization patterns are nearly identical with those of human nephrin. Like the human, the rat nephrin transcript is expressed in a tissue restricted pattern. Thus, preparation of transgenic animals are enabled by the present invention.

Experimental models of diabetic and other nephropathies and diabetes mellitus and other inflammatory and neoplastic pancreatic diseases are important tools to investigate the functional significance of novel molecules by providing a means to effectively modulate the basic functions. Thus, even if transgenic and knockout mouse models are extremely useful in the targeted analysis of molecules, the well established experimental models in the rat remain best characterized and most widely used for demonstrating pathophysiology of diabetic and other nephropathies and diabetes mellitus and other inflammatory, immunologic and neoplastic pancreatic diseases. Thorough understanding of this gene encoding nephrin and nephrin-like protein molecules as well as its regulation and functions are imperative also for developing targeted therapeutic actions.

Circulating Antinephrin Antibodies in Human ANF Patients

The novel gene NPHS1 is defective in the patients with congenital nephrotic syndrome of the Finnish type (CNF) leading to abnormal expression of the protein nephrin in glomerular cells. CNF patients are treated with early nephrectomy and renal transplantation, but about 20% patients have developed nephrosis. The mechanism for this is unknown. Indirect immunofluorescence microscopy and an ELISA assay was used to search for circulating autoantibodies to nephrin, the protein product missing in the majority of Finnish CNF patient kidneys. In serial serum samples gathered before and after renephrosis an increased antibody titer prior to the renephrosis episode and a subsequent drop in antibody level after a successful treatment of the renephrosis was shown. The results show that the transplantation treatment introduces a neoantigen inducing production of autoantibodies, which subsequently perturb the function of the glomerular filtration barrier. Based on said results convenient and rapid methods and test kits can be developed and be used to check the success of therapies and/or surgical intervention.

CNF patients with renephrosis

Detailed characteristics of CNF patients with renephrosis including HLA matching, cytomegalovirus and Epstein-Barr virus infection history, clinical course before and after renephrosis, histologic analysis, description of the clinical pre- and post-transplantation and renephrosis treatments as well as analysis of the kidney biopsies have been reported earlier in detail (Laine, et al. Kidney International 44: 867-874, 1993). Briefly, there was no overrepresentation in male/female ratio, donor source, acute rejection or septic infections or significant HLA-A and-B mismatches in the renephrosis patients, and blood

cyclosporine concentration was within target limit. Serum creatinine concentration had increased slightly since the previous hospital visit. Serum albumin and protein concentrations were characteristically low and all patients had over protenuria (for details, see (Laine, et al. Kidney International 44: 867-874, 1993). The complete laboratory findings of urine and serum of the renephrosis patients have been reported earlier (Laine, et al. Kidney International 44: 867-874, 1993).

For demonstrating the accuracy and effectiveness of the present invention optimization of the ELISA assay was achieved by using different concentrations of the coating peptide and by preincubation of the patient sera with the competitive oligopeptides, respectively. Also the controls of the second and irrelevant antibody reactivities with or without coating peptides were negative. Optimization of the ELISA also included the reactivity with antibodies to synthetic nephrin-specific sequences. These antibodies reacted with the typical double band at the 185 kDa area (Fig. 8). Consecutive patient serum samples taken after renal transplantation before the renephrosis showed systematic elevation of antibody levels (Fig. 4), while sera from CNF patients without renephrosis failed to show such an elevation. After successful treatment of the renephrosis episode with steroids, cyclophosphamide and cyclosporin (for the regimen, see Kaine, et al. Kidney International 44:867-874, 1993), the antibody titres of the individual patients decreased within 1-3 months for both the intracellular and extracellular antibodies. These results show that circulating antinephrin antibodies are formed in human subjects and are associated with damage to the kidney filtration barrier.

Nephrin regulation by protein kinase _C (PKC)_

Quantitation of nephrin mRNA expression by RT-PCR

The A293 early human embryonic kidney cells earlier found suitable for expression study of nephrin was used in order to study the nephrin mRNA regulation in detail. Remarkable nephrin regulation was observed particularly with phorbol-12-myristate-13-acetate (PMA). The mRNA expression of nephrin increased by 340% in PMA group, while ocadaic acid (OA) and bradykinin showed negligible upregulation to nephrin by 125% and 107%, respectively as compared to the level of ß-actin, while lysophosphatidic acid (LPA) decreased mRNA level of nephrin to 82% (Table 1).

Table 1
Quantification of nephrin mRNa in cultured A293 cells

Stimulants	24 hours	48 hours
Control	1.00 ± 0.28	1.00 ± 0.24
+ OA 1 nM	1.18 ± 0.17	1.25 ± 0.21
+ PMA 100 nM	1.40 ± 0.27	3.40 ± 0.34*
+ LPA 58 nM	0.95 ± 0.14	0.82 ± 0.17
+ Bradykinin 1 μM	0.98 ± 0.22	1.07 ± 0.24

The values were normalized according to ß-actin level.
* $p < 0.01$; NS, no significant difference. (n = 3)

In time-course experiments of (PMA) stimulation, no appreciable changes were seen at 2, 4, 8, 12 hour, whereas at 24 hour nephrin-specific mRNA started to increase and was at maximum at 48 hour.

Nephrin expression in cultured cells
In order to compare the mRNA expression with its corresponding protein product, we used polyclonal antibody to nephrin to stain the cells after stimulation. No obvious changes were found after LPA, OA and bradykinin treatment respectively (data not shown). However, consistantly after PMA stimulation, the intensity of staining was stronger than in control cells.

$[Ca^{2+}]_i$ in CNF and normal human cells after different stimulants
In CNF and normal human glomerular cells, $[Ca^{2+}]_i$ was measured by microfluorometry of cells loaded with the intracellular fluorescent probe, fura 2. Baseline $[Ca^{2+}]_i$ in CNF and normal cells equilibrated in nominally Ca^{2+}-free media was 74.6 ± 8.4 and 69.4 ± 4.1 respectively (Table 2). Upon graded addition of extracellular Ca^{2+}, $[Ca^{2+}]_i$ increased.

Table 2

$[Ca^{2+}]_i$ in glomerular cells from normal subject (NS) and congential nephrotic syndrome (CNS) after different stimulation

Stimulants	NS	CNS
Baseline $[Ca^{2+}]_i$, nM	69.4 ± 4.1	74.6 ± 8.4
+ Ca^{2+} 1mM	199 ± 25	162 ± 20
+ Ca^{2+} 10mM	425 ± 29	379 ± 61
+ ANG II 1μM	783 ± 145	647 ± 112
+ AVP 0.1μM	n.r.	822 ± 52
+ Ca^{2+} 1mM, ANG II	353 ± 38	284 ± 38
+ Ca^{2+} 10mM, ANG II	666 ± 102	557 ± 69

Data are mean ± SE nmol/L peak $[Ca^{2+}]_i$ from n = 5-7 monolayers for each cell line; n.r., not responsive

Changes of nephrin expression in experimental diabetic nephropathy

The molecular pathogenesis of glomerular diseases with proteinuria is poorly understood while increasing evidence suggests a key role for the podocytes in the permeability changes. Kestilä et al. (Mol Cell 1:575-582,1998) recently cloned the gene NPHS1 mutated in the massively proteinuric congenital nephrotic syndrome of the Finnish type (CNF) patients. It has been shown that the respective protein product, nephrin, localizes mainly to the interpodocyte filtration slit area, while we could show that some nephrin is always present at the lateral podocyte membranes. Our results have further shown distinct alternative splicing of nephrin-specific mRNA and typical alteration of nephrin mRNA levels during experimental renal diseases in parallel to loss of nephrin into urine.

Since diabetic nephropathy is a major cause of glomerular injury, scarring and proteinuria nephrin participation in the molecular pathogenesis of this group of diseases was taken as the object for our studies. For this purpose, the classic streptozotocin (STZ) model of experimental diabetes of the rat was used. STZ induces an insulin-dependent diabetes mellitus (IDDM) by destroying insulin production in the ß-cells of the islets of Langerhans in the pancreas. In addition, the spontaneously diabetic

non-obese diabetic (NOD) mice were studied. The spontaneous development of diabetes in NOD mice is caused by an autoimmune destruction of the ß-cells.

Albuminuria in STZ rats started at week 4 after induction (mean 0.47 g/l) and increased rapidly by six (mean 5.5 g/l) and sixteen weeks (mean 5.6 g/l) in spite of the daily injections of insulin. Free nephrin as detected by immunoblotting was found in the urine of STZ rats already at four weeks and was at maximum at six weeks. Interestingly, in real-time RT-PCR (TaqMan^R) the production of nephrin-specific mRNA increased by 200 to 500% in the models of IDDM studied here. Immunohistochemical study showed a distinct change of nephrin localization within glomeruli in the STZ rats: the typical podocyte pattern of reactivity at the cells facing the urinary space to a more central localization was found suggesting of accumulation of nephrin around the glomerular capillaries.

Together these results show that changes in nephrin mRNA and protein levels and localization are associated with the initial stages of the loss of glomerular permeability barrier in diabetic nephropathy. Importantly, similar changes were observed in both the toxic (STZ) and autoimmune (NOD) models. The early loss of nephrin into urine is interesting and suggests that the splice variant yielding soluble nephrin is involved.

Changes in nephrin protein in human kidney diseases results

Normal tissue1.
All normal kidneys were negative for the 18C7 antibody, both in glomeruli and interstitium. No reactivity with other human tissues was observed.

Human kidney biopsis
Among the 100 kidney biopsies stained with the 18C7 antibody, 48 were completely negative both at glomerular and interstitial level. Glomerular positivity was found in 52 biopsies: the staining was always parietal, lining the glomerular basement membrane with different degrees of intensity and diffusion (Figure 6). The immunohistochemical results obtained in different renal diseases are shown in Table 3.

Among all diseases, a 2+ glomerular positivity was detected in membranous nephropathy, membranoproliferative glomerulonephritis, systemic erythematous lupus (all class IV), and cryoglobulinemic nephritis.

Table 3
Renal biopsies diagnosies and 18c7 staining

Diagnosis	Number of cases			
	18c7 = 0	18c7 = 0.5	18c7 = 1	18c7 = 2
Mesangioproliferative glomerulonephritis - IF negative	0	1	1	0
Acute post-streptococcal nephritis	1	0	1	0
Minimal change disease	2	1	0	0
Primary focal and segmental glomerulosclerosis	0	1	2	0
Membraneous nephropathy	2	6	6	6
IgA glomerulonephritis	26	5	2	0
Membranoproliferative glomerulonephritis	1	3	0	2
Henoch-Schoenline syndrome	4	2	0	0
Systemic erythematous lupus	2	2	0	5
Cryoglobulinemic glomerulonephritis	1	0	1	2
Renal vasculitis	0	2	0	0
Anti-GBM glomerulonephritis	0	0	1	0
Amyloidosis	2	0	0	0
Nephroangiosclerosis	2	0	0	0
Acute interstitial nephritis	2	0	0	0
Acute tubular necrosis	1	0	0	0
Alport syndrome	2	0	0	0
Total	48	23	14	15

Among the 39 cases of primary and secondary IgA nephropathy, that was the widest diagnostic group, most biopsies were negative (30 cases) and among the 9 positive cases, none showed a 2+ positivity.

Table 4 illustrates the results obtained comparing clinical and histological parameters with different levels of staining. Although an increasing trend in proteinuria, segmental glomerular sclerosis, extracapillary proliferation and glomerular leukocyte infitration was accompanying the different levels of 18c7 positivity, no statistical difference could be found among the groups. Instead, the statistical analysis disclosed a significant association between 18c7 positivity and thickness of the glomerular basement membrane ($P \leq 0.04$).

Table 4

Comparison of clinical and histological features according to 18c7 expression

	Serum creat (mg/dl)	Urinary proteins (g/day)	Global glomerular sclerosis (%)	Segmental glomerular sclerosis (%)	Mesangial proliferation (0+, 1+, 2+)	GBM thickness (0+, 1+, 2+)	Necrosis (%)	Extracapillary proliferation (%)	Glomerular leukocyte infiltration (no.CD45+cells/mm²)
18c7=0 (no.=48)	1.6±1.6	2.3±4.9	15±10	4±2	0.8±0.6	0.2±0.6	0	3±8	4.6±6.1
18c7=0.5 (no.=23)	1.9±1.4	3.6±3.4	16±10	6±4	1±0.6	0.4±0.6	4±8	5±12	6.4±8.5
18c7=1 (no.=14)	1.7±1.6	3.3±2.3	14±8	6±3	0.7±0.8	0.7±0.8	2±6	8±10	6.6±9.6
18c7=2 (no.=15)	1.5±0.5	5.2±3.3	18±11	7±5	1.2±1	1.4±0.5	0	13±16	12.4±32.2
P-value 0 vs 0.5	ns	ns	ns	ns	ns	ns	ns	ns	ns
P-value 0 vs 1	ns	ns	ns	ns	ns	0.04	ns	ns	ns
P-value 0 vs 2	ns	ns	ns	ns	ns	0.002	ns	ns	ns
P-value 0.5 vs 1	ns	ns	ns	ns	ns	ns	ns	ns	ns
P-value 0.5 vs 2	ns	ns	ns	ns	ns	0.005	ns	ns	ns
P-value 1 vs 2	ns	ns	ns	ns	ns	0.03	ns	ns	ns

Moreover, in 13 cases with different degrees of glomerular positivity, some endothelial staining was detected, mostly localized at the vascular pole of the glomerulus, but also present in some interstitial small sized vessels (Fig 5). Dividing biopsies according to presence or absence of endothelial staining, no statistical significance was obtained for any clinical and histological parameter. Figures 5A and 5B show normal findings and Figures 5C and 5D show diseased kidney.

Nephrin protein expression shows remarkable changes in various groups of human glomerular disease: both up/down-regulation and new expression patterns within the glomerulus. Whether these changes lead to loss of circulating nephrin into urine and thus available for early diagnostics is currently under study.

The present invention and its feasibility for different usages is illustrated in more detail in the examples below. The examples should not be used to restrict the scope of the protection in any way. Those skilled in the art can based on these example develop a multitude of methods and test kits and screening programs for producing new drugs and treatment modalities.

Example 1

Antipeptide antibodies to nephrin

Design of synthetic peptides.

Sequence specific intracellular (aa 1101-1126) and extracellular (aa 1039-1056) oligopeptides were selected over the human nephrin sequence (Gene bank accession number AF035835) using the PredictProtein program via Internet at European Molecular Biology Laboratory, (Heidelberg, Germany). These peptides showed no homology to other known protein sequences and were synthesized and purified at a local peptide synthesis unit (Haartman Institute, University of Helsinki).

Example 2

Antipeptide antibodies

For immunizations the peptides were coupled to a multiple antigenic peptide-polylysine matrix) and injected in two rabbits each. First immunization was with 500 μg of peptide in Freunds complete adjuvant (Difco laboratories, Detroit, Mi), and two booster immunizations with 300 μg each with Freunds incomplete adjuvant four weeks after the previous immunization. Peptide specific fractions were immunoaffinity purified on CNBr-Sepharose (Pharmacia, Uppsala, Sweden) coupled to the corresponding linear

peptides. The specificity of the antisera was tested by immunofluorescence (IF) on kidney sections with and without free peptide competition (Fig. 1a), by immunoblotting of glomerular extracts and precipitation of a full length nephrin in an *in vivo* transcription and translation assay using the specific antibodies (see below).

Example 3

Electrophoresis and Western blotting.

For SDS-PAGE the detergent extracts of isolated human glomeruli were suspended in the Laemmli sample buffer, boiled for 5 min, and run under reducing conditions using 8 % gels and a Protean Mini-gel electrophoresis system (Bio-Rad Laboratories, Richmond, CA). The separated proteins were transferred to nitrocellulose sheets for Western blotting using a Novablot semidry blotting apparatus (Pharmacia). After blocking with 3 % BSA the nitrocellulose strips were incubated with the respective antibodies, washed thoroughly and further incubated with anti-rabbit IgG coupled to horseradish peroxidase. After washing the bound antibodies were detected using the ECL^{TM} Western blotting kit (Amersham Pharmacia Biotech).

Example 4

Immunofluorescence and immunoelectron microscopy.

Samples from normal human cortical tissue were prepared for immunofluorescence. Briefly, frozen cortical tissues were cut at 4 μm, fixed in acetone at -20 °C for 10 min and reacted with the antipeptide antibodies (1.1 mg/ml; used at 1:100 dilution in PBS) for 1 hour. FITC-antirabbit IgG (Boehringer, Mannheim, Germany) was used as second antibodies. As control, the primary antibodies were either omitted or replaced by irrelevant antipeptide antibodies. An additional control included preincubation of the antibody with a dilution series of the oligopeptide used as the original immunogen. Postembedding electron microscopy was done using CNF and normal kidney cortical kidney samples fixed in freshly prepared 4% formaldehyde in PBS and embedded in Lowicryl K4M (Chemische Werke LOW1, Waldkraiburg, Germany) and further incubated with the rabbit antinephrin antibodies (50 μg/ml) and the respective 10 nm gold conjugate (1:50).

Example 5

***In vitro* transcription/translation.**

In vitro transcription/translation of full length nephrin sequence under T3 promoter of pBK-CMV (Promega, Madison, WI) was performed according to the manufacturer's instructions with TNT T3 Coupled Reticulocyte Lysate System (Promega, Madison, WI)

- a single tube modification of rabbit reticulosyte lysate translations. During translation nephrin was labeled with [^{35}S]methionine (NEN Life Science Inc., Boston, MA). Intracellular antibodies (see above) and [^{35}S]labeled nephrin were incubated without or with increasing amounts (0.1, 1 and 10 μg) of intracellular peptide overnight at 4 °C. Immunocomplexes were collected with protein A - Sepharose (Zymed Laboratories Inc. San Francisco, CA) by incubating immunocomplexes and protein A-Sepharose 45 minutes at 4 °C. After incubation the immunocomplexes were washed eight times with washing buffer (150 mM NaCl, 20 mM TRIS (pH 7.6), 0.15% Tween 20, 0.1% BSA, 0.02% sodium azide), and the radioactivity of precipitates was measured in scillation liquid OptiPhase SuperMix (EG&G Wallac, Finland) with 1450 MicroBeta Trilux Liquid Scillation&Luminescence Counter (EG&G Wallac, Finland).

Example 6

Immunoprecipitation.

For immunoprecipitation the glomerular lysate (1 mg/ml) in RIPA buffer was incubated with rabbit or mouse antibodies against occludin (Zymed Laboratories Inc., San Francisco, CA) or ZO-1 (Zymed) using 10 μg of IgG/200 μl of glomerular lysate at 4 °C overnight. Immune complexes were collected with protein-A-Sepharose (Pharmacia LKB Biotechnology), washed and processed for immunoblotting with antinephrin antibodies as described above.

Example 7

Mutation analysis.

Analysis of the Fin_{major} (in exon 2) and Fin_{minor} (exon 26) mutations in the only patient sample out of 28 studied showing immunoreactivity with the antinephrin antibodies was done. Briefly, after DNA isolation, the respective exon areas were amplified by PCR using AmpliTaq DNA polymerase (Perkin Elmer) and the following conditions: initial denaturation at 94 °C for 12 min, 30 PCR cycles (95 °C 1 min, 60 °C 1 min and 72 °C 1 min) followed by final elongation at 72 °C for 8 min. The primer sequences for exon 2 were 5'-GAC AAA GCC AGA CAG ACG CAG-3' (SEQ ID NO:6:) and 5' AGC TTC CGC (SEQ ID NO:7:).

Example 8

Characterization of the gene and the gene product

Conventional immunohistochemical methods, in-situ hybridization and Northern blotting, and well as semiquantitative RT-PCR and electron microscopy can be performed with

methods already available and previously used. Our glomerulus-pancreas-gene and its product can be used as a novel pathogenic epitope of kidney diabetic complications, being an imperative factor for a thorough understanding of this gene, its regulation and functions are imperative also for developing targeted therapeutic actions.

Example 9

Analysis of autoantibodies; ***Autoantibodies to gene product:***

Determination and characterization of autoantibodies in diabetes and proteinuria.

The already existing first results of autoantibody production in diabetic patients will be expanded systematically and includes an analysis of antibodies in the sera available from the DiMe Research Consortium.

Another source for expanding the patient sera derives from the accumulating samples for the analysis of islet cell (ICA) and glutamic acid dehydrogenase (GAD65) antibodies at HUCH-Diagnostica (500-800 samples/year). Particularly, the data so far show that 45-60 % of the elevated ICA and GAD positive sera are positive with our kidney- pancreas-epitope, and a careful analysis of patient data will be needed to characterize this patient group. Also, the outcome of diabetic patients treated with kidney transplantation and analyzing their antibody levels will be of utmost importance.

Example 10

Expression of nephrin mRNA in human tissues.

Expression of nephrin mRNA in 50 different human tissues was studied with the Human RNA Masterblot (Clontech, Palo Alto, USA). After hybridization with the human nephrin probe, a clear hybridization signal was observed only in adult (square E1 in the Masterblot) and fetal kidney (G3) as well as in the pancreas (D3) (Figure 6).

Example 11

Northern blot. Presence of nephrin mRNA was studied in human pancreas and kidney cortex with the Northern blot method. This revealed a 5 kb band in kidney cortex as well as in the pancreas. The hybridization signal was stronger in the kidney cortex. When normalized with the ß-actin (B) signal the difference was at least 2.5 fold.

Example 12

Reverse Transcriptase PCR (RT-PCR).

With RT-PCR, nephrin and α-nephrin mRNA were found in isolated islets of Langerhans (lanes 2 and 6), human pancreas (lanes 3 and 7), as well as in human kidney

cortex (lanes 4 and 8). With the primer pair 2606U/2979L upstream from putative transmembrane region of human nephrin (Fig. 1) a single product of an expected size (374 bp) was obtained (lanes 2 4) (Fig. 7). The other primer pair with the primer 3094U upstream and the primer 3402L downstream of the exon 24 encoding the transmembrane region showed two bands (lanes 6 8) one of which was approximately 310 bp representing the whole nephrin sequence between primers and another of about 190 bp. This size corresponds precisely to the expected product size of the spliced α-nephrin without the transmembrane sequence. The absence of the exon 24 (120 bp) was confirmed by sequencing. The RT- control reactions showed no PCR product. All ß-actin reactions gave the expected PCR product (350 bp).

Example 13

Sequencing

The nephrin mRNA between bases 1 and 4285 was sequenced from human pancreatic cDNA library with nephrin primer pairs. The sequence was found to be exactly the same in pancreas and kidney. Also sequencing from the pancreatic cDNA library confirmed the existence of a splicing variant mRNA, α-nephrin.

Example 14

Western blot

In Western blot the nephrin intracellular antibody detected 185 and 165 kDa protein bands in human kidney cortex (lane 1) but only a 165 kDa band in human pancreas (lane 2) (Fig. 9). The 165 kDa two-band pattern is more visible in lane 3 where the pancreas lysate with the extracellular E2 antibody. Lane 4 shows the immunoblotting result of extracellular E1 antibody in the pancreas lysate. All three antibodies detected the same protein band in pancreas. However, in kidney the 185 kDa band was better visualized with the intracellular antibody and extracellular E1 antibody than wi th the extracellular E2 antibody (data not shown). The extracellular part has several glycosylation sites, which may affect the recognition and binding of the E2 monoclonal antibody produced against the E2 core protein without glycosylation. The controls stained with rabbit serum, IgG or mouse IgM gave no signals.

Example 15

Immunohistochemistry

Paraffin sections of mouse pancreas were stained with the intracellular (Fig. 9A and Fig. 9B), extracellular E1 (Fig. 9C) and E2 (Fig. 9D) antibodies. All three nephrin antibodies specifically stained islets of Langerhans, Successive sections incubated with

the intracellular nephrin (Fig. 9A and 9B) and insulin antibodies (Fig. 9E and 9F) led to the staining of identical cells, thus localizing the immunoreactive nephrin in the ß-cells. Double staining with glucagon and nephrin E1 antibodies (Fig. 9C) showed the typical perpheral glucagon staining in the islets (dark purple) while nephrin-positive cells (brownish red) were localized centrally. Preliminary results showed increased levels of circulating autoantibodies to nephrin as measured with ELISA assay in subgroups of islet-cell antibody (ICA) and glutamic acid decarboxylase (GAD65) antibody positive prediabetic patient sera. Together these results suggest that nephrin may be particularly involved in the pathophysiology of also diabetic kidney disease, the most important complication of insulin dependent diabetes mellitus.

Example 17

Detection of circulating autoantibodies

In the group of ICA positive sera the mean absorbance was 0.387 (range 0.073 - 1.133). The mean absorbance of the GAD65 positive sera was 0.242 (range 0.066 - 0.928). In comparison, the mean absorbance value of 11 normal sera was 0.183 (range 0.067 - 0.339). The secondary antibody control (background) gave an absorbance of less than 0.1. The mean absorbance, median 0.0178, standard deviation 0.07). To divide the ICA GAD positive samples (n=27) into nephrin antibody positive and negative groups. We used cutoff limit mean absorbance value of normal sera +2SD(0.327). With this criterion ten samples of the 27 ICA and GAD positive samples (41%) were regarded as positive and 17 samples (59%) negative. 53% of the ICA positive samples had antinephrin autoantibodies while only 3 (25%) GAD positive sera showed them.

Example 18

RT-PCR

To detect the expression level of rat nephrin in mRNA in experimental models we used semiquantitative RT-PCR as Northern blotting was not sensitive enough. For this purpose we chose the nucleotides (2692-2994) near the transmembrane area. In puromycin nephritis, a 40% downregulation at postinjection day 3 and 80% downregulation at day 10 was observed as compared to the respective levels in the controls. With $HgCl_2$, a closely similar pattern, rapid decrease of nephrin mRNA level was seen, whereas pretreatment with probucol resulted in a mild increase to 1.2 and 1.4 time of the level of control at day 3 and 10, respectively. No changes were observed in the group treated with paclitaxel. In the group of compining PAN and $HgCl_2$ treatment an even more rapid drop in the nephrin level was observed already at day 3. In the

paclitaxel-pretreated group, no appreciable drop was seen at day 3, and the drop at day 10 was only 38% as compared to the basal level.

Example 19

Immunohistochemistry

Antibodies against the intracellular domain of nephrin showed a typical exclusive localization within glomeruli in the normal kidneys. An altered pattern of reactivity was seen in the PAN treated groups: a more patchy and less intense (from +++ to +/++) was seen in the 10 day samples whereas negligible changes were seen at day 3. A similar change in the $HgCl_2$ group was seen. No difference to controls was seen in the other experimental groups.

Example 20

Immunoelectron microscopy

In postembedding immunoelectron microscopy the anti-intracellular nephrin antibodies labeled distinctly the interpodocyte areas of normal rat glomeruli at the level of the filtration slits, while practically no gold particles were seen outside the podocytes. However, especially in the diseased glomerulus, single gold particles were regularly observed along the apical plasmamembrane of podocytes while even in these situations most of the label as observed at the infiltration slits.